Published in final edited form as:

Thromb Haemost. 2010 November 3; 104(5): 990–997. doi:10.1160/TH10-03-0161.

Hypodysfibrinogenaemia due to production of mutant fibrinogen alpha-chains lacking fibrinopeptide A and polymerisation knob 'A'

Silja Vorjohann¹, Richard J. Fish¹, Christine Biron-Andreani², Chandrasekaran Nagaswami³, John W. Weisel³, Pierre Boulot⁴, Lionel Reyftmann⁴, Philippe de Moerloose⁵, and Marguerite Neerman-Arbez^{1,5}

¹ Department of Genetic Medicine and Development, University of Geneva Medical Centre, Geneva, Switzerland ² Hematology Laboratory, University Hospital, Montpellier, France

³ Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA ⁴ Department of Gynecology and Obstetrics, University Hospital, Montpellier, France ⁵ Division of Angiology and Hemostasis, University Hospital and Faculty of Medicine, Geneva, Switzerland

Summary

Inherited disorders of fibrinogen are rare and affect either the quantity (hypofibrinogenaemia and afibrinogenaemia) or the quality of the circulating fibrinogen (dysfibrinogenaemia) or both (hypodysfibrinogenaemia). Extensive allelic heterogeneity has been found for all these disorders: in congenital afibrinogenaemia for example more than 40 mutations, the majority in *FGA*, have been identified in homozygosity or in compound heterozygosity. Numerous mutations have also been identified in patients with hypofibrinogenaemia, many of these patients are in fact heterozygous carriers of afibrinogenaemia mutations. Despite the number of genetic analyses performed, the study of additional patients still allows the identification of novel mutations. Here we describe the characterization of a novel *FGA* intron 2 donor splice-site mutation (Fibrinogen Montpellier II) identified in three siblings with hypodysfibrinogenaemia. Functional analysis of RNA produced by the mutant minigene in COS-7 cells revealed that the mutation led to the in-frame skipping of exon 2. Western blot analysis of COS-7 cells expressing an exon 2 deleted *FGA* cDNA revealed that an alpha-chain lacking exon 2, which codes in particular for fibrinopeptide A and polymerisation knob 'A', has the potential to be assembled into a hexamer and secreted. Analysis of precipitated fibrinogen from patient plasma showed that the defect leads to the presence in the circulation of alpha-chains lacking knob 'A' which is essential for the early stages of fibrin polymerisation. Fibrin made from purified patient fibrinogen clotted with thrombin displayed thinner fibers with frequent ends and large pores.

Keywords

Hypodysfibrinogenaemia; fibrinogen assembly; fibrinogen secretion; mutation identification and expression

Introduction

The final step in the coagulation cascade is the conversion of fibrinogen to fibrin which polymerises and creates the basis of the blood clot. Fibrinogen is a 340 kDa glycoprotein predominantly synthesised in the liver and composed of two sets of three homologous polypeptide chains known as A α -, B β -, and γ chains, which are encoded by distinct genes, *FGA*, *FGB*, and *FGG*, respectively, clustered in a region of 50 kb on chromosome 4q31 (1). In the endoplasmatic reticulum (ER) the chains are assembled to form a hexameric structure (A α B β γ)₂ and a signal peptide (19 amino acids for A α , 30 for B β and 26 for γ) is co-translationally removed in the secretory pathway (2). Upon activation of the coagulation cascade, fibrin is produced by proteolytic cleavage of the fibrinogen alpha and beta chains by thrombin, thus releasing fibrinopeptides A and B and allowing polymerisation to occur (3).

Normal plasma fibrinogen levels vary between 1.5 and 3.5 g/l. Inherited disorders of fibrinogen are rare and affect either the quantity (hypofibrinogenaemia and afibrinogenaemia) or the quality of the circulating fibrinogen (dysfibrinogenaemia) or both (hypodysfibrinogenaemia).

More than 400 cases of dysfibrinogenaemia have been reported to date, the first dysfibrinogenaemia mutation being identified as early as 1968 (4). The majority of dysfibrinogenaemias are caused by missense mutations in one of the three fibrinogen genes. Missense mutations at residue R35, which is part of the thrombin cleavage site in the fibrinogen alpha chain, are the most common (5). Dysfibrinogenaemias are most often asymptomatic but can cause bleeding, thrombosis or both (5–7).

While congenital afibrinogenaemia is characterised by complete deficiency of fibrinogen, hypofibrinogenaemia is diagnosed when functional and antigenic fibrinogen levels fall below a concentration of 1.5 g/l. Although in the past hypofibrinogenaemia was considered a separate disorder, with both dominant and recessive modes of inheritance proposed, we and others have shown that in many cases patients are asymptomatic and are in fact heterozygous for null mutations which in homozygosity or compound heterozygosity would cause afibrinogenaemia (8–9). The majority of causative mutations that have been characterised to date are located in the *FGA* gene (8–11).

Here we describe the characterisation of a novel heterozygous mutation in the *FGA* gene, (Fibrinogen Montpellier II) identified in three siblings with reduced functional fibrinogen. We found an insertion of three nucleotides close to the donor splice site after exon 2 and upon analysing the impact on the splicing process in COS-7 cells we detected an aberrant mRNA product missing exon 2. With these results and the clinical findings that suggested the presence of non-functional fibrinogen in patient plasma, we tested if the mutant alpha-chain can be translated, assembled and secreted in a cellular model and how effective this process is in comparison with the wild-type chain. Finally, fibrinogen purified from the three siblings was analysed and their clot structure examined.

Patients and methods

Patients

Two sisters of white European descent, aged 25 and 23, were investigated for hypo-(dys)fibrinogenaemia following either obstetrical complications or thrombosis, respectively. Their younger asymptomatic brother aged 18 years was also included in the study. Informed consent was obtained from all three individuals, the parents were unavailable for this study.

Mutation screening

Genomic DNA was extracted from fresh blood samples in EDTA using standard procedures. The exons and intron-exon junctions of the *FGA* gene of the probands' DNA were amplified by polymerase chain reaction (PCR) and sequenced as previously described (10). After identification of the causative mutation, the probands' sister and brother were then genotyped for the mutation.

Minigene constructs

A 4 kilobase pair (kb) PCR product containing *FGA* wild-type and mutant sequences were obtained directly from genomic DNA of the proband by PCR-amplification.

Oligonucleotides were situated in the 5' prime untranslated region and exon 5 of the *FGA* gene (forward primer FGAX1L: CAGCCCCACCCTTAGAAAAG; reverse primer FGAX5R: GCGGCATGTCTGTTAATGCC) and a standard PCR-reaction using the Herculanse Hot Start DNA polymerase (Stratagene, La Jolla, CA, USA) was used. The 4 kb PCR product was cloned into the pcDNA3.3-TOPO-TA expression vector (Invitrogen, Groningen, The Netherlands). Plasmid DNA preparations were purified from individual clones and sequenced to identify wild-type and mutant sequences.

Transfection of COS-7 cells and RT-PCR analysis

Cos-7 cells were cultured in DMEM-10% fetal calf serum (FCS) and passaged using standard procedures. Plasmids were transfected using FuGENE HD Transfection Reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol. Briefly, 3 µg of either the wild-type or the mutant genomic *FGA*-construct were transfected next to a non-transfected control receiving only the transfection reagent. RNA was extracted 48 hours (h) later with the RNeasy kit (Qiagen, Basel, Switzerland) and reverse transcription performed with Superscript II (Invitrogen, Groningen, The Netherlands) using random hexamer primers (Promega, Wallisellen, Switzerland). The cDNA served as template in a PCR with the FGAX1L and FGAX5R primers, which were used in the construction of the synthetic gene expression plasmid. PCR-products were sequenced to compare the wild type and mutant open reading frames (ORFs).

cDNA expression plasmids

To investigate whether the exon 2 deleted alpha-chain is expressed in cells and is able to be incorporated into a fibrinogen hexamer, a cDNA expression plasmid was constructed. This required the removal of 42 codons, corresponding to *FGA* exon 2, from the wild-type cDNA sequence. Overlap extension PCR (12) was used to generate the modified cDNA using a wild-type *FGA* cDNA construct as template which has been described previously (13). Briefly, a 5' PCR product was amplified which spans from upstream of the *FGA* cDNA in the pcDNA3.1 expression plasmid to the site of the mutated splice junction. The reverse oligonucleotide has complementary nucleotides to the region downstream of the deletion at its 5' (FGAdelx2R:

GCAGCCAGAAGGGCATTGTAGTTCCATGCTGTGCCCACCACACTTAG). A second PCR spanned from downstream of the mutation site into the expression plasmid at the 3' of the cDNA. The forward primer for this reaction has nucleotides from upstream of the mutation site in its 5' (FGAdelx2F:

CTAAGTGTGGTGGGCACAGCATGGAACCTACAAATGCCCTTCTGGCTGC). The resulting products were purified, mixed and a third PCR was performed to assemble the complete mutated cDNA using the mixed PCR products as template (forward primer T7: TAATACGACTCACTATAGGG and reverse primer BGHr:

TAGAAGGCACAGTCGAGG were used). The mutated *FGA* cDNA was confirmed by DNA sequencing.

Study of fibrinogen assembly and secretion in COS-7 cells

For protein analysis, the fibrinogen wild type or mutant FGA cDNA expression vectors were transfected into COS-7 cells with wild-type FGB and FGG expression plasmids, as described previously (13). At 24 h after transfection, cells were washed with phosphate buffered saline (PBS) and incubated for an additional 24 h in media without serum. Cell lysates and conditioned media were harvested and Western blot analyses were carried out as described previously (13–14).

Fibrinogen analysis

Fibrinogen was purified by precipitation from plasma with 20% saturated ammonium sulphate as described by Brennan et al. (15–16). After standing at 4°C for 30 minutes the precipitate was collected by centrifugation and washed twice with 20% saturated ammonium sulphate before being dissolved in water at 37°C. Western blot analyses were carried out as described previously (13–14).

Scanning electron microscopy of control and mutant fibrin clots

Fibrinogen was dialyzed into 50 mM Tris-HCl, 0.15 M NaCl, pH 7.4 and the protein concentration was adjusted so that the protein in all clots was about equal, approximately 1 mg/ml. Clots were formed in small chambers by addition of thrombin, 0.5 NIH U/ml, and were allowed to clot for 1 h at room temperature in a moist environment. The clots were washed with 50 mM Na-Cacodylate buffer, pH 7.3, fixed with 2% glutaraldehyde in the same buffer, and then prepared by a modification of a previously described procedure (17). The samples were dehydrated in a graded series of ethanol solutions through 100% over a period of several hours. After the 100% ethanol step, samples were immersed in graded strengths of hexamethyldisilazane (HMDS) (Acros Organics, Fair Lawn, NJ, USA) in ethanol and finally in 100% HMDS three times, followed by air-drying overnight in a fume hood (18). Then, samples were mounted on aluminium stubs using carbon tape and sputter-coated with gold palladium. Clots were examined with a Phillips/FEI XL20 scanning electron microscope (FEI Company, Hillsboro, OR, USA) at 10 KV accelerating voltage. Several areas of clots were visualised in an unbiased manner and digital images were taken at high resolution.

Results

Family characteristics, clinical history and laboratory data

A 25-year-old woman was referred to our Haemostasis Unit for suspicion of a coagulation disorder when a prolonged prothrombin time (PT) was observed during routine screening before a surgical procedure (coelioscopy). The patient described no notable bleeding history: no easy bruising, no epistaxis, no menorrhagia, no bleeding after minor surgery (tooth extraction). There was no personal history of thrombosis. Her father had previously been diagnosed with a low level of fibrinogen during presurgical coagulation studies, in his case, follow-up studies of the underlying cause were not undertaken. There was no other case of thrombosis in the family. No family member reported excessive bleeding.

A full coagulation evaluation revealed a functional fibrinogen level (Clauss method) of 66 mg/dl (normal range, 150–350 mg/dl) and an immunologic level of 122 mg/dl (normal range, 150–350 mg/dl), a normal platelet count (178 G/l) and normal levels of factors II, V, VII, VIII, IX and XI. Congenital hypodysfibrinogenaemia was diagnosed by discrepancy between the functional and immunologic fibrinogen levels. Thrombophilia screening was negative: antithrombin activity was 85 % (normal range 80–130%), protein C activity was 92% (normal range 65–130%), protein S activity was 55% (normal range 50–130%); factor

V Leiden, factor II G20210A polymorphism, lupus anticoagulant, anti-cardiolipin and anti- β 2 glycoprotein I antibodies were all negative.

Shortly after diagnosis she became pregnant with her first child. At 19 weeks' gestation she was admitted in emergency for metrorrhagia. An ultrasound showed a blood collection. Rapidly, she developed uterine contractions and persistent bleeding. Placental abruption with a disseminated intravascular coagulation was diagnosed that required infusion of fibrinogen concentrates, fresh frozen plasma and blood products. Therapeutic abortion was performed. The patient has since had a successful second pregnancy, under fibrinogen replacement therapy without any haemorrhagic or thrombotic complication.

The proband's sister, aged 23, was also diagnosed with low clottable fibrinogen (80 mg/dl) when thrombophilia screening was performed for a first proximal venous thrombosis associated with oral contraceptive use. Recently, she developed a second proximal deep-vein thrombosis associated with a pulmonary embolism after a long journey and an infectious disease (diarrhea and dehydration). Their younger brother also has low clottable fibrinogen (60 mg/dl) but is asymptomatic.

Genetic analysis and genotype/phenotype correlation

Previous studies on the characterisation of mutations leading to fibrinogen deficiencies showed that in most cases, the mutations are found in *FGA*. On this basis we chose to screen this gene first and found a novel mutation (Fibrinogen Montpellier II) in the exon-intron junction of exon 2 in *FGA*: all three family members with low clottable fibrinogen were heterozygous for an insertion of three nucleotides (CAT) between nucleotides +3 and +4 of intron 2 (IVS2+3insCAT or c.180+3_+4insCAT according to the nomenclature recommended by the Human Genome Variation Society) (Fig. 1). Since the disruption of the sequence occurs close to the donor splice site and converts the conserved purine at the third position of the donor site into a pyrimidine, we wished to determine if the mutation caused aberrant mRNA splicing. Computer splice prediction analysis of the region around the IVS2 donor site with the program Splice-view (19) detected in the normal sequence only the physiological donor site with a score of 84 (functional splice sites have scores between 75 and 85). In the presence of the IVS2+3 ins CAT mutation, the physiologic site could no longer be detected and no new cryptic splice sites were predicted by the simulation. We therefore hypothesised that the donor site is no longer functional in the mutant allele and leads either to a truncated, shortened mRNA or a longer version including intron 2.

To test this hypothesis, genomic wild-type and IVS2+3 ins CAT vector constructs encompassing exons 1 to 5 of *FGA* were cloned as described in *Patients and methods* and were used to transiently transfect COS-7 cells. RNA was extracted after two days and analysed by RT-PCR. Only one product was visible by gel electrophoresis for each construct: the wild-type RT-PCR product obtained was of the expected size (786 bp) whereas the mutant product was approximately 130 bp smaller than the wild-type (Fig. 2). Sequencing of the cDNAs showed that transcription of the mutant construct lead to the skipping of 126 base pairs corresponding to the complete exon 2 in the spliced mRNA.

Further analysis of the mutant sequence showed that the aberrant splicing of the mRNA did not introduce an internal stop codon and the sequence was still in frame. Exon 2 encodes the last amino acid of the signal peptide followed by fibrinopeptide A and the 'A knob' which is essential for the first steps of fibrin polymerisation (3, 20). Analysis of the new signal peptide generated by the fusion of exon 1 and exon 3 amino acid residues using Signal P (21) predicted that the signal peptide would be functional, with the most likely cleavage site occurring between residues 19 and 20, as for the wild-type signal peptide. In addition, domains responsible for assembly and secretion of the hexamer are most likely unaffected

by the mutation. Indeed, in a study investigating the involvement of N-terminal domains in fibrinogen assembly (22), deletion of 41 amino acids from the N-terminus of the alpha-chain did not impair secretion of single alpha-chains, nor assembly and secretion of the fibrinogen hexamer in transfected COS cells. We therefore tested if the mutant FGA mRNA is translated and the resulting polypeptide chain, missing amino acids 1–41 of the mature chain (or 19–60, counting from the initiator Met) is assembled into the fibrinogen hexamer and secreted *in vitro*.

To express an FGA chain missing exon 2, an overlap extension PCR was made on a FGA cDNA construct (see *Patients and methods*) and the mutant FGAX2del cDNA construct was transiently co-transfected in COS-7 cells with wild-type FGB and FGG cDNAs. To mimic the heterozygous state, one transfection experiment received the normal FGA cDNA construct in addition to the mutant vector. At 48 h after transfection, individual fibrinogen chains (under reducing conditions) or assembled hexamers (under non-reducing conditions) in cell extracts and conditioned media were detected by Western blot analysis with a polyclonal antifibrinogen antibody. Co-transfection with the three normal cDNAs showed expression of all three polypeptides, normal assembly inside the cell, and adequate secretion of the hexamer (Fig. 3). When co-transfections were performed with wild-type FGB and FGG cDNAs, and with the mutant FGAX2del cDNA, the three polypeptides were expressed, and the truncated FGA chain could be detected in the cell extracts as well as in the conditioned media. However, the band appears to be slightly fainter, especially after co-transfection with the wild-type chain, which indicates that either the expression efficiency or the stability of the mutant chain is reduced.

We found that the mutant FGA polypeptide can be successfully incorporated into a hexamer and secreted into the media, even in the presence of the normal FGA polypeptide. Consistent with the results obtained for the individual polypeptide chains, the signal detected for the intracellular and secreted mutant hexamer appears to be lower as compared to the wild-type.

The results of this *in vitro* study, and the clinical phenotype (hypodysfibrinogenaemia rather than hypofibrinogenaemia), suggested that fibrinogen containing alpha-chains missing the amino acids encoded by exon 2 should be present in the patient's plasma. Fibrinogen was precipitated from the index patients' plasma, as well as from the plasma of her siblings, and analysed via Western blot as described above. Compared to a purified fibrinogen control and fibrinogen precipitated from plasma of a healthy control individual, a shorter alpha-chain could be detected under reducing conditions in the samples from all three siblings (Fig. 4). Compared to the wild-type band, the mutant chain was less abundant, consistent with a smaller fraction of mutant chains incorporated into the successfully secreted hexamer. No mutant hexamer could be detected due to the small size difference of 41 amino acids for each alpha-chain, which is unresolved in the non-reducing conditions used. There is an apparent discrepancy between the low level of mutant alpha-chain detected and the levels of clottable fibrinogen measured for the patients, which is approximately half of the total fibrinogen. One likely explanation for this result is that while the mutant chain is expressed at similar levels to the wild-type chain, the polyclonal antibody used does not recognise the mutant alpha-chain as well. Use of a monoclonal antibody specific to fibrinopeptide A, such as the monoclonal Y18 antibody developed by Koppert et al. in 1985 (23), which would not react with the mutant chain in Western blots would have allowed us to estimate the relative amounts of mutant and normal alpha-chains in the plasma samples of our patients. However this antibody was not available for our study. Alternatively, low levels of aberrant alpha-chain, present in homodimers but mostly in heterodimers may be sufficient to perturb the clotting process even if the majority of fibrinogen molecules produced are wild-type.

Clots were prepared by addition of thrombin to purified fibrinogen from control and patients and they were examined by scanning electron microscopy (Fig. 5). While control clots were made up of a uniform network of branching fibres with few fibre ends visible (Fig. 5A), the patient clots were non-uniform, with clusters of generally thinner fibres and large pores, with many fibre ends visible (Fig. 5B–D). The clots from the index patient (Fig. 5B) were clearly more abnormal than those from her sister (Fig. 5C) and brother (Fig. 5D).

Discussion

We identified a novel heterozygous *FGA* splice-site mutation in three siblings i.e. an insertion of three bases at the donor splice site of intron 2. Functional analysis revealed that the mutation induced exon 2 skipping and the resulting mutant alpha-chain had the potential to be assembled into a hexamer and secreted. Interestingly the three siblings have the same heterozygous genotype and low functional fibrinogen but have different clinical manifestations: the proband had a bleeding complication of pregnancy (placenta abruptio), her sister had two venous thromboembolic events (albeit in a context of oral contraceptive usage and smoking) while their brother is asymptomatic. This situation is encountered for several mutations identified in dysfibrinogenemia, for which environmental factors and genetic polymorphisms have been shown to modulate the phenotype of the disorder (5). The difference in phenotype could also be explained by the relative levels of the mutant alpha-chain in the circulation of the three siblings. Our data obtained using purified fibrinogen suggest that this is not the case since similar amounts of the aberrant alpha-chain were detected for all three individuals. However it would be interesting to determine more precisely the ratio of variant to normal alpha-chain in our patients.

Conversion of fibrinogen to a fibrin clot occurs in three phases: i) enzymatic cleavage by thrombin produces fibrin monomers; ii) fibrin monomers self-assemble to form an organised polymeric structure and iii) factor XIIIa cross-links fibrin. In the first phase of conversion to fibrin, cleavage of fibrinogen at A α R35/G36 (R16/G17 without the signal peptide) and later B β R44/G45 (R14/G15 without the signal peptide) results in release of fibrinopeptides A (FpA) and B (FpB), respectively, thus exposing knobs 'A' and knobs 'B' (3, 24). The knobs 'A' located at the new amino-terminal end of the fibrin alpha-chain starts with the GPRV amino acid sequence. The knob 'A' in fibrin interacts with the constitutive complementary association site known as hole 'a' in the γ C domain in another molecule to initiate the fibrin assembly process. Knobs 'A' – holes 'a' interactions result in formation of double-stranded fibrils (protofibrils) in which fibrin molecules become aligned in an end-to-middle, staggered overlapping arrangement.

The presence of an aberrant alpha-chain lacking the amino acids of FpA and the knobs 'A' induces detrimental effects on fibrin polymerisation, as demonstrated in clots observed by scanning electron microscopy (Fig. 5). The aberrant alpha-chain identified in this study is incapable of carrying out the first steps of fibrin polymerization, which are driven by knobs 'A' – holes 'a' interactions. Thus, the clottability of this fibrinogen is greatly reduced, indicating that some of the protein is incapable of being incorporated into the polymer. In addition, the clot that is formed, especially for the fibrinogen from the index patient, is quite abnormal, with clusters of thin fibres and fibre ends, with large pores. It could be that homodimers containing two abnormal A α chains do not polymerise, while heterodimers, with one abnormal and one normal chain, can be incorporated into a growing protofibril, but form a cap on one end of the protofibril, because there is only one knob 'A'. This would explain the appearance of the clots with clusters of thin fibres, fibre ends and large pores that arise because the fibres can not grow longer because of the capped ends. It should be noted that this abnormal clot structure is similar to that observed for fibrinogen Hershey III, which has an A α R35C (R16C) substitution, precluding cleavage of FpA (25). In contrast to knobs

'A' – holes 'a' interactions, knobs 'B' interactions with the holes 'b' situated in the β C domain are not absolutely required for protofibril formation and are weaker but probably enhance lateral aggregation (26–28). When knobs 'A' – holes 'a' interactions are impaired, for example by introducing mutations in hole 'a', knobs 'B' – holes 'b' interactions allow thrombin-catalysed polymerisation of uniform, ordered fibrin clots with fibres thicker than normal (28). In our patients where knobs 'A' – holes 'a' interactions are impaired by a naturally occurring mutation that eliminates knob 'A', it is possible that knobs 'B' – holes 'b' interactions are contributing to the polymerisation of fibrin molecules containing the mutant alpha-chain. It is not known why the clots from fibrinogen of the index patient are more abnormal than those from her sister's and brother's fibrinogen, although there could be differing amounts of abnormal chains or other polymorphisms or post-translational modifications that are not the same in all three siblings. However, it may be significant that the most striking clinical manifestations, of bleeding in the index patient, correspond to the most abnormal clot structures observed. Furthermore, the clinical history of bleeding is consistent with such an abnormal clot structure with many fibre ends and large gaps.

Interestingly, another mutant fibrinogen alpha-chain lacking fibrinopeptide A and part of the 'A' knob has been previously reported due to a completely different underlying molecular mechanism (15). Fibrinogen Canterbury, identified in a patient with prolonged thrombin time and mild bleeding tendency, is the result of a heterozygous missense mutation in *FGA* i.e. V39D (V20D). The mutation introduces an aberrant proprotein cleavage site at residue R38 (R19) which is recognised by furin, resulting in the removal of fibrinopeptide A and the GPR amino acid residues of the 'A' knob.

Finally, a mutation similar to the one identified here has been described in *FGB* in a family with dysfibrinogenaemia and a history of thrombosis (29): fibrinogen New York I is characterised by deletion of amino acids 9–72 in the mature beta-chain (amino acids 39–102 from the initiator Met), corresponding to residues encoded by *FGB* exon 2. Because the aberrant fibrinogen was characterised at the time by Edman degradation sequencing, the exact mutation from the DNA point of view is not known. Fibrinogen New York I lacks the C-terminal residues of fibrinopeptide B and the 'B' knob starting with GHRP. The authors suggested that the lack of amino acids encoded by *FGB* exon 2 destabilises the structure of the N-terminal region of the alpha-chain. Indeed, residue beta C95(C65) forms a disulfide bond with C55(C36) of the alpha-chain, its absence in Fibrinogen New York I is likely to cause a conformational change in the N-terminal region of the abnormal fibrinogen due to the free SH group in the alpha-chain. In our study, residues encoded by *FGA* exon 2 are missing including C55(C36) which probably cause structural changes in the mature protein due to the free SH group in the beta-chain. Since the quality and structural integrity of fibrinogen is controlled before secretion (27), these structural changes may impair secretion of the mutant, which could explain the lower levels of the mutant chain compared to wild-type we observed both in the transfected COS7 cell model and in the plasma of the three heterozygous individuals. However, further studies on the structure of the mutant protein are necessary to confirm this.

Acknowledgments

We are grateful to Corinne di Sanza for expert technical assistance. We thank all family members for their participation in this study.

References

1. Kant JA, Fornace AJ Jr, Saxe D, et al. Evolution and organization of the fibrinogen locus on chromosome 4: gene duplication accompanied by transposition and inversion. *Proc Natl Acad Sci USA*. 1985; 82:2344–2348. [PubMed: 2986113]

2. Redman CM, Xia H. Fibrinogen biosynthesis. Assembly, intracellular degradation, and association with lipid synthesis and secretion. *Ann NY Acad Sci.* 2001; 936:480–495. [PubMed: 11460506]
3. Mosesson MW, Siebenlist KR, Meh DA. The structure and biological features of fibrinogen and fibrin. *Ann NY Acad Sci.* 2001; 936:11–30. [PubMed: 11460466]
4. Blomback M, Blomback B, Mammen EF, et al. Fibrinogen Detroit--a molecular defect in the N-terminal disulphide knot of human fibrinogen? *Nature.* 1968; 218:134–137. [PubMed: 5645286]
5. Hanss M, Biot F. A database for human fibrinogen variants. *Ann NY Acad Sci.* 2001; 936:89–90. [PubMed: 11460527]
6. Haverkate F, Samama M. Familial dysfibrinogenemia and thrombophilia. Report on a study of the SSC Subcommittee on Fibrinogen. *Thromb Haemost.* 1995; 73:151–161. [PubMed: 7740487]
7. Galanakis DK. Inherited dysfibrinogenemia: emerging abnormal structure associations with pathologic and nonpathologic dysfunctions. *Semin Thromb Hemost.* 1993; 19:386–395. [PubMed: 8140431]
8. Maghazal GJ, Brennan SO, Homer VM, et al. The molecular mechanisms of congenital hypofibrinogenemia. *Cell Mol Life Sci.* 2004; 61:1427–1438. [PubMed: 15197468]
9. Neerman-Arbez M, de Moerloose P. Mutations in the fibrinogen gene cluster accounting for congenital afibrinogenemia: an update and report of 10 novel mutations. *Hum Mutat.* 2007; 28:540–553. [PubMed: 17295221]
10. Neerman-Arbez M, de Moerloose P, Bridel C, et al. Mutations in the fibrinogen alpha gene account for the majority of cases of congenital afibrinogenemia. *Blood.* 2000; 96:149–152. [PubMed: 10891444]
11. Asselta R, Duga S, Tenchini ML. The molecular basis of quantitative fibrinogen disorders. *J Thromb Haemost.* 2006; 4:2115–2129. [PubMed: 16999847]
12. Ho SN, Hunt HD, Horton RM, et al. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene.* 1989; 77:51–59. [PubMed: 2744487]
13. Neerman-Arbez M, Germanos-Haddad M, Tzanidakis K, et al. Expression and analysis of a split premature termination codon in FGG responsible for congenital afibrinogenemia: escape from RNA surveillance mechanisms in transfected cells. *Blood.* 2004; 104:3618–3623. [PubMed: 15284111]
14. Vu D, de Moerloose P, Batorova A, et al. Hypofibrinogenemia caused by a novel FGG missense mutation (W253C) in the gamma chain globular domain impairing fibrinogen secretion. *J Med Genet.* 2005; 42:e57. [PubMed: 16141000]
15. Brennan SO, Hammonds B, George PM. Aberrant hepatic processing causes removal of activation peptide and primary polymerisation site from fibrinogen Canterbury (A alpha 20 Val --> Asp). *J Clin Invest.* 1995; 96:2854–2858. [PubMed: 8675656]
16. Brennan SO, Davis RL, Chitlur M. New fibrinogen substitution (gammaSer313Arg) causes diminished gamma chain expression and hypodysfibrinogenemia. *Thromb Haemost.* 2010; 103:478–479. [PubMed: 20126833]
17. Weisel JW, Nagaswami C. Computer modeling of fibrin polymerization kinetics correlated with electron microscope and turbidity observations: clot structure and assembly are kinetically controlled. *Biophys J.* 1992; 63:111–128. [PubMed: 1420861]
18. Nation JL. A new method using hexamethyldisilazane for preparation of soft insect tissues for scanning electron microscopy. *Stain Technol.* 1983; 58:347–351. [PubMed: 6679126]
19. Rogozin IB, Milanesi L. Analysis of donor splice sites in different eukaryotic organisms. *J Mol Evol.* 1997; 45:50–59. [PubMed: 9211734]
20. Weisel JW. Fibrinogen and fibrin. *Adv Protein Chem.* 2005; 70:247–299. [PubMed: 15837518]
21. Emanuelsson O, Brunak S, von Heijne G, Nielsen H. Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Protoc.* 2007; 2:953–971. [PubMed: 17446895]
22. Zhang JZ, Redman CM. Assembly and secretion of fibrinogen. Involvement of amino-terminal domains in dimer formation. *J Biol Chem.* 1996; 271:12674–12680. [PubMed: 8647881]
23. Koppert PW, Huijsmans CM, Nieuwenhuizen W. A monoclonal antibody, specific for human fibrinogen, fibrinopeptide A-containing fragments and not reacting with free fibrinopeptide A. *Blood.* 1985; 66:503–507. [PubMed: 4040783]

24. Blomback B, Hessel B, Hogg D, et al. A two-step fibrinogen--fibrin transition in blood coagulation. *Nature*. 1978; 275:501–505. [PubMed: 692730]
25. Flood VH, Nagaswami C, Chernysh IN, et al. Incorporation of fibrin molecules containing fibrinopeptide A alters clot ultrastructure and decreases permeability. *Br J Haematol*. 2007; 138:117–124. [PubMed: 17555455]
26. Litvinov RI, Gorkun OV, Galanakis DK, et al. Polymerization of fibrin: Direct observation and quantification of individual B:b knob-hole interactions. *Blood*. 2007; 109:130–138. [PubMed: 16940416]
27. Geer CB, Tripathy A, Schoenfisch MH, et al. Role of 'B-b' knob-hole interactions in fibrin binding to adsorbed fibrinogen. *J Thromb Haemost*. 2007; 5:2344–2351. [PubMed: 17892530]
28. Okumura N, Terasawa F, Haneishi A, et al. B:b interactions are essential for polymerization of variant fibrinogens with impaired holes 'a'. *J Thromb Haemost*. 2007; 5:2352–2359. [PubMed: 17922804]
29. Liu CY, Koehn JA, Morgan FJ. Characterization of fibrinogen New York 1. A dysfunctional fibrinogen with a deletion of B beta(9–72) corresponding exactly to exon 2 of the gene. *J Biol Chem*. 1985; 260:4390–4396. [PubMed: 3156856]

Figure 1. Identification of a novel mutation at the donor splice site of *FGA* intron 2

A) Partial sequence of *FGA* showing the insertion of CAT in heterozygosity in the patient.
 B) Control and mutant IVS2+3 ins CAT constructs used for functional analysis. The genomic *FGA* constructs which were obtained by PCR amplification from the patient's DNA, contain the complete coding sequences from exons 1 to 4, complete introns 1 to 4, and part of exon 5. The haplotype specific SNPs were maintained.

Figure 2. Functional analysis of the ins CAT mutation at the donor splice site of *FGA* intron 2
 A) RT-PCR of COS-7 cells transfected with wild-type and mutant (IVS2+3 ins CAT) *FGA* minigene constructs. Only a single band is visible for each construct: the wild-type RT-PCR product obtained (Wt) is of the expected size (786 bp) whereas the mutant product (Mut) is approximately 130 bp smaller. B) Partial sequences of Wt and Mut RT-PCR products. The mutation leads to the in-frame skipping of exon 2.

Figure 3. Western blot analysis of cell extracts and conditioned media of COS-7 cells transfected with fibrinogen cDNAs

Samples of cell lysates and culture medium were subjected to 9% SDS-PAGE under reducing conditions (A) or 7% SDS-PAGE under non-reducing conditions (B). The blots were incubated with a polyclonal antihuman fibrinogen antibody. FG, purified fibrinogen control; NT, non-transfected COS-7 cells; wt, COS-7 cells transfected with normal A α , B β and γ cDNAs; mut, COS-7 cells transfected with normal B β and γ cDNAs plus mutant FG α 2del cDNA; mut/wt: COS-7 cells transfected with normal B β and γ cDNAs and a mix (0.5 μ g mutant/0.5 μ g normal) of alpha-chain cDNAs. The results show that the exon 2-deleted alpha-chain is expressed in the COS-7 cells and, at least in this model system, is capable of forming a hexamer which is secreted into the cell medium.

Figure 4. Western blot analysis of precipitated fibrinogen from control and patients plasma
 Samples were subjected to 7% SDS-PAGE under non-reducing conditions (A) or 9% SDS-PAGE under reducing conditions (B). The blots were incubated with a polyclonal antihuman fibrinogen antibody. FG indicates purified fibrinogen control; PC, fibrinogen precipitated from control plasma of a healthy individual; lanes PP1, 2 and 3 correspond to fibrinogen precipitated from plasma of the index patient, her sister and her brother, respectively. The two bands of the alpha-chain visible in all the samples correspond to the complete A α form and the truncated chain: the additional band detected for the proband and her siblings corresponds to the mutated form missing amino acids 1–41 of the mature chain (or 19–60, counting from the initiator Met).

Figure 5. Scanning electron microscope images of control and mutant fibrin clots

Fibrin clots made by addition of thrombin to purified fibrinogen from the index patient, her sister and brother are generally made of thinner fibres with fibre ends, resulting in clusters of fibres with some pores in the mesh-work, where fibres end. Clots from Control (A), Index patient (B), her sister (C) and her brother (D). Magnification bar = 10 μ m.